Graphene-Based Materials in Regenerative Medicine

Xili Ding, Haifeng Liu, and Yubo Fan**

Graphene possesses many unique properties such as two-dimensional planar structure, super conductivity, chemical and mechanical stability, large surface area, and good biocompatibility. In the past few years, graphene-based materials have risen as a shining star on the path of researchers seeking new materials for future regenerative medicine. Herein, the recent research advances made in graphene-based materials mostly utilizing the mechanical and electrical properties of graphene are described. The most exciting findings addressing the impact of graphene-based materials on regenerative medicine are highlighted, with particular emphasis on their applications including nerve, bone, cartilage, skeletal muscle, cardiac, skin, adipose tissue regeneration, and their effects on the induced pluripotent stem cells. Future perspectives and emerging challenges are also addressed in this Review article.

1. Introduction

Graphene is a single-atom thick, two-dimensional sheet of sp^2-hybridized carbon atoms, which has received much interest in the field of materials physics, chemistry, science, and biotechnology since the few-layers graphene (FLG) was isolated from its three-dimensional parent material, graphite.[1–4] Graphene possesses remarkable physical-chemical properties, including high fracture strength, high Young's modulus, excellent thermal and electrical conductivity, large specific surface area, and biocompatibility due to its unique structure and geometry.[2,5] In recent years, graphene has attracted much attention for numerous potential applications in biomedicine, such as disease diagnostics, antibacterial and antiviral materials, biosensing, cancer targeting and photothermal therapy, electrical stimulation of cells, drug delivery, and tissue engineering.[2–6] These various applications have attracted the interest in manufacturing not only graphene monolayers but also graphene-related materials including FLG, graphene nanosheets, graphene oxide (GO), and reduced GO (rGO), which can be included in graphene family nanomaterials (GFNs) (as shown in **Figure 1**).[7,8]

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Although graphene has been widely used in electronic and chemical fields, its applications in regenerative medicine still have significant room for improvement. Studies on tissue regeneration have been stimulated by the need for improved treatments. Moreover, in combination with stem cell technology, appropriate materials for directing stem cell differentiation have now become much more critical for tissue regeneration. It is well known that graphene consists of a layer with π-conjugated structure of six-atom rings, which can be conceptually viewed as a planar aromatic macromolecule. The planar structure imparts graphene the excellent capability to immobilize a large number of substances which include drugs, metals, fluorescent probes, biomolecules, and cells.[9–13] The mechanical properties of graphene play a key role in the context of tissue engineering due to the highest Young's modulus among any known materials.[14,15] Moreover, graphene-modified substrates can be easily bent into any needed shape.[16] On the other hand, conductive graphene can be used as fillers with insulating polymer matrix to enhance the electrical conductivity of the composites. Graphene-based materials with good electrical properties can be used for cell electrical stimulation as well. Therefore, it is not surprising that graphene has generated great interest in regenerative medicine.

To describe the significance of graphene, we investigate the publications and the increase of citation rates using Web of Science (date of search: February 13, 2015). The articles including the words of "graphene" and "tissue engineering" are counted in the period of 2005–2015. **Figure 2**A,B shows the growth rate of publications and citations in all these years. The analysis of the publication data reveals that the largest number of studies have been carried on graphene-based materials for bone and nerve tissues regeneration (Figure 2C). This tendency clearly shows the global importance of graphene and the increasing interest of scientists in this area. Obviously, graphene-based materials have risen as a shining star on the path of researchers seeking for new materials applied in future regenerative medicine. This review highlights recent research advances made in graphene-based materials, with particular emphasis on their applications in regenerative medicine.

2. Concept and Challenges

2.1. Graphene Family Nanomaterials

GFNs are classified based on either their chemical modification or number of layers in the sheet. The widely used GFNs include

single layer graphene, FLG, GO, and rGO. Each member of GFNs varies in layer number, surface chemistry, lateral dimensions, purity, composition, and defect density.[17]

Single layer graphene has caused the most interest for its unique electronic properties.[2] It can be synthesized by repeated mechanical exfoliation of graphite flakes,[18] or controlled grown on substrates via chemical vapor deposition (CVD).[19] Pristine graphene of significant lateral dimension is difficult to synthesize. In addition, it is very hard to be suspended in solvents due to its reactive surface. Therefore, much attention has been paid on multi-layer graphene and GO in biological applications.

FLG is defined as flake-like stacks of 2–10 graphene layers, which is initially produced as a byproduct of the fabrication of monolayer graphene.[4] Nitrate, sulfate, or other ions are introduced between the layers of graphite and then are treated by rapid thermal heating, which gives rise to tremendous expansion and a buildup of internal pressure of the layered structure of graphite. This thermal exfoliation will produce dry powders that can be dispersed into FLG samples or further processed into graphene or GO.[7]

GO is generated by severe oxidation of graphite followed by sonication to generate a single-layer material. GO consists of single atom thick layer of graphene sheets with carboxylate groups on the periphery. The carboxylate groups will provide colloidal stability and pH dependent negative surface charge.[20] Hydroxyl and epoxide functional groups present on the basal plane are uncharged but polar. Unmodified graphitic domains are also contained on the basal planes and they are hydrophobic and capable of π–π interactions, which can adsorb some drugs and dry molecules. Therefore, GO is an amphiphilic sheet-like macromolecule and it can act like a surfactant to collect at interfaces or stabilize hydrophobic molecules in a solution.[21,22] High defect density is produced by the presence of functional groups in GO, which leads to reduce its mechanical, electrical, and thermal properties.[23]

rGO can be obtained by treatment of GO under reducing conditions, which include high-temperature thermal treatment and chemical treatments with hydrazine or other reducing agents.[20] The aim of reduction of GO is to restore electrical conductivity. However, it increases hydrophobicity and reduces oxygen content because of CO/CO_2 liberation.[24] In addition, it reduces water dispersibility and surface charge.[7]

2.2. Toxicity and Biocompatibility

Before using graphene-based materials in clinical, it is vital to investigate their biocompatibility and toxicity by in vitro and in vivo studies. Compared with spherical nanoparticles, nanotubes, or nanorods, the structure of graphene is unique and we know less about the toxicity of graphene-based materials and their interactions with cells.[7] The issues about the safety and toxicity of graphene-based materials have not been thoroughly resolved, thereby much attention needs to be paid to evaluate their biocompatibility and toxicity.

The interactions between graphene or GO sheets and target cells including lung epithelial cells,[25] fibroblasts,[26] and neural cells[27] have been studied to evaluate their toxicity. It was demonstrated that monolayer GO sheets were internalized and sequestered by human lung epithelial cells or fibroblasts. The in vitro results showed that the monolayer GO sheets could induce toxicity when the doses were above 20 μg/mL after 24 h.[26,28] The effects of GO on A549 cells were also found to be dose related.[29] A minimal toxic dose was 50 μg/mL, while the extracellular generation of reactive oxygen species (ROS) was discovered at high concentrations of GO. In another study, Zhang et al. reported that FLG induced mitochondrial injury and

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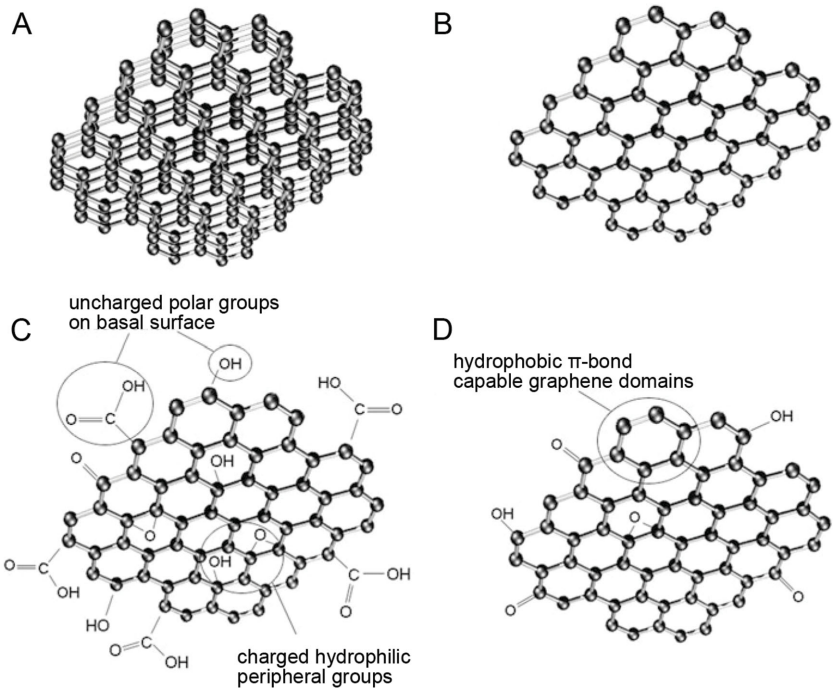

Figure 1. A schematic diagram of the graphene family nanomaterials: A) FLG, B) graphene nanosheet, C) GO, and D) rGO. Adapted with permission.[8] Copyright 2012, Springer.

increased intracellular generation of ROS in neural cells after 4 and 24 h at a dose of 10 μg/mL.[30] In addition to the dependence of toxicity on the dose of graphene-based materials, their sizes can influence the toxicity as well. The size effect of GO in response to different types of cells was systematically evaluated by Yue et al.[31] and the results showed that the micro-sized GO induced much stronger inflammation responses compared with the nano-sized GO. Along similar lines, Makharza et al.[32] found that GO samples with average widths of 200 and 300 nm exhibited a cytotoxic effect on mesenchymal stem cells (MSCs) and HeLa cells, whereas the GO samples with an average width of 100 nm showed no significant cytotoxicity. Size is also an important factor affecting the distribution of GO after intravenous administration. The investigation of the GO size on organ distribution and accumulation showed that the micro-sized GO was trapped in the lungs, nevertheless, the submicro-sized GO could pass across the vascular tissue and thereafter accumulated in the liver.[33] Moreover, surface modification of graphene had been demonstrated to alter its toxicity,[34] while rGO and carboxylated graphene were shown to be less toxic than GO or pure graphene.[35]

Different from the above-mentioned studies in which graphene-based nanomaterials were added into the cell cultures in solution, researchers had also developed graphene-based substrates to investigate their biocompatibility and toxicity.[36] For example, a study by Agarwal et al.[37] indicated that films made from a suspension of rGO were non-cytotoxic to three different types of mammalian cells,such as neuroendocrine PC12 cells, oligodendroglia cells, and osteoblasts. In another study, colorectal adenocarcinoma (HT-29) cells showed marked cell enlargement and spreading on the GO films, which clearly indicated that the GO films exhibited no toxicity to the cells and could be a great support for mammalian cell attachment, growth, and proliferation.[38] Furthermore, Li et al. fabricated a 3D porous graphene foam by CVD method as a scaffold for neural stem cells (NSCs) culture in vitro. It was found that the 3D graphene foams could not only support NSC growth, but also keep cell at an active proliferation state with upregulation of Ki67 expression than that of 2D graphene films.[39]

There are few toxicological studies using animal models, which indicates that the development and applications of graphene are still in the early stage.[40] For example, Kunming mice were intravenously administered single-layered GO sheets of 10–800 nm in lateral size at a dose of 1 mg/kg or 10 mg/kg.[41] After 14 days post-injection, no pathological alterations were observed with the lower dose. However, it was found that pulmonary oedema, inflammation, and fibrosis were triggered by the increased accumulation of graphene in the lungs and its slow clearance with 10 mg/kg dose. In order to look into the biocompatibility of GO after intravenous administration, Wang and colleagues injected three different doses of GO to Kunming mice. Although no signs of toxicity were found with the low and medium doses, the high dose exposure showed chronic toxicity, which was caused by inflammatory response in the lung as well as the formation of lesions and granulomas.[42]

Polyethylene glycol (PEG) has been widely used to functionalize carbon nanotubes and other nanomaterials to improve their biocompatibility.[43,44] PEG was successfully attached onto GO by Dai and co-workers for drug delivery applications. The exfoliation prepared PEG-GO composites exhibited an excellent stability in physiological solutions, which were shown in **Figure 3**A.[45] Several in vitro studies showed that coating GO with PEG could exhibit inappreciable toxicity to many cell lines, even at high concentrations above 100 mg/L.[45,46] Furthermore, Yang et al. functionalized nanographene sheets with PEG coated by fluorescent labels and investigated their behaviors in mice by in vivo fluorescence imaging. Forty days after treatment, no obvious sign of toxicity for PEGylated graphene injected mice was revealed (Figure 3B–D).[47] This study confirmed that the toxicity of graphene was closely related to its functionalization, which was in agreement with previous studies on CNTs.[48] In another study, Duch et al. explored hybridization strategies to improve the biocompatibility of graphene nanomaterials.[49] They indicated that the pulmonary toxicity was mainly caused by the covalent oxidation of graphene, and the toxicity was significantly reduced in the case of the homogeneous distribution of unoxidized graphene in the Pluronic.

The quantitative in vivo biodistribution especially long-term toxicity study of implanted graphene-based materials using a more accurate and reliable method is urgently demanded in

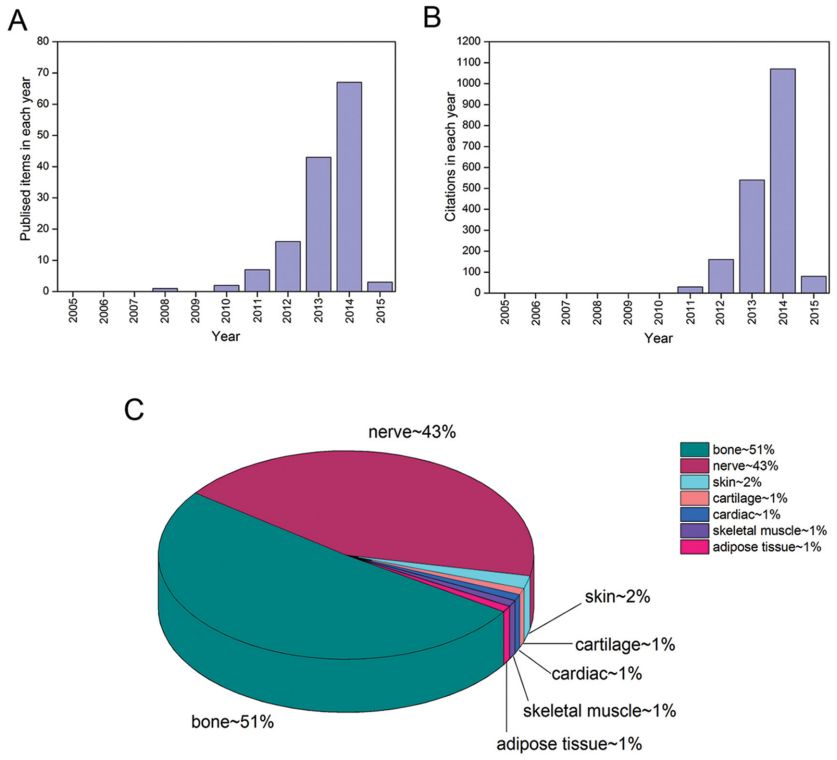

Figure 2. A,B) Publication and citation trend in the field of graphene-based tissue regeneration (Data obtained from Web of Knowledge, Thomson Reuters; search strings: "graphene" and "tissue engineering"). C) Relative percentage of reports being published on graphene-based in vitro engineering of various tissues (Data obtained from Web of Knowledge, Thomson Reuters; search strings: "graphene" and "tissue engineering").

this field. Yang and colleagues for the first time studied the in vivo long-term biodistribution of PEGylated graphene.[50] They reported that PEGylated nanographene sheets mainly accumulated in the reticuloendothelial system including spleen and liver after intravenous administration, which could be gradually cleared without causing any appreciable toxicity over a period of 3 months. However, the long-term safety of the graphene-based materials needs further observation over a longer period of time. As well, a lot more systematic explorations are required to fully understand the in vivo long-term fate and toxicology of graphene-based materials in various animal models before they can be translated into the clinic.

3. Graphene-Based Materials

3.1. Utilization of the Mechanical Properties of Graphene

Graphene is one of the strongest materials since the breaking strength of monolayer defect-free graphene is about 200 times higher than steel.[51] Fracture strength, Young's modulus, and Poisson's ratio for defect-free graphene are 130 GPa, 1 TPa, and 0.149 GPa, respectively.[52] However, the mechanical properties of GO are dramatically lower than that of pure graphene.[53,54] For example, the fracture strength and elastic modulus of paper-like layered GO platelets are 120 MPa and 32 GPa, respectively.[17,55] Therefore, graphene has been used as fillers or reinforcements in films, electrospun fibers, 3D porous scaffolds, and hydrogels to improve the mechanical properties of polymeric materials, which means the potential applications of graphene in tissue regeneration.

To investigate the reinforcing behavior of graphene sheets in the graphene-reinforced biocompatible materials, graphene/chitosan (CS) films were fabricated by solution casting method.[56] The results showed that the elastic modulus of CS increased after being added a small amount of graphene. It is important to have the uniform filler dispersion within the polymer matrix and good interfacial adhesion between polymer matrix and nanofillers. Thus, nanocomposites of CS and GO were prepared by simple self-assembly of both components in aqueous media, which indicated good dispersion of GO sheets within the nanocomposites.[57] Along similar lines, Pan et al. presented a simple and green approach to fabricate CS films reinforced with parallel aligned GO.[58] The tensile modulus and fracture strength of the nanocomopsites were improved, which could be attributed to the strong interfacial adhesion between GO and CS, the homogeneous dispersion and alignment of GO sheets in the CS matrix. Moreover, Depan et al. incorporated GO into CS scaffolds by covalently linking the amine groups of CS with the carboxyl groups of GO, which could form a network structure scaffold with improved mechanical properties (**Figure 4**A,B).[59]

As the "click chemistry" technique attracted much attention from the scientists, Ryu et al. utilized it for realizing the covalent attachment of alkynyl-decorated GO with azide-moiety-containing CS with excellent mechanical properties.[60] The graphene sheets could have strong interactions with the polymer matrix and be well dispersed in the CS matrix. With the addition of a small amount of the click coupled CS functionalized graphene in the CS matrix, the tensile modulus and breaking stress of the CS composite films were increased by over 200%.

Composite films of silk fibroin (SF) and GO sheets with layered structures were fabricated by facile solution casting of SF-GO hydrogels.[61] The excellent mechanical properties of this film were attributed to the strong hydrogen bonding interactions between GO sheets and SF chains. Similarly, Gopiraman et al. fabricated cellulose acetate (CA) hybrid nanofibers with graphene and graphene-COOH via electrospinning technique.[62] Faghihi et al. evaluated the effects of GO nanosheet content on the linear and nonlinear mechanical properties of poly(acrylic acid) (PAA)/gelatin hydrogels.[63] The tensile strength and elongation at break of composite hydrogels were significantly increased by the addition of GO nanosheet. These graphene-based composites with high strength and the

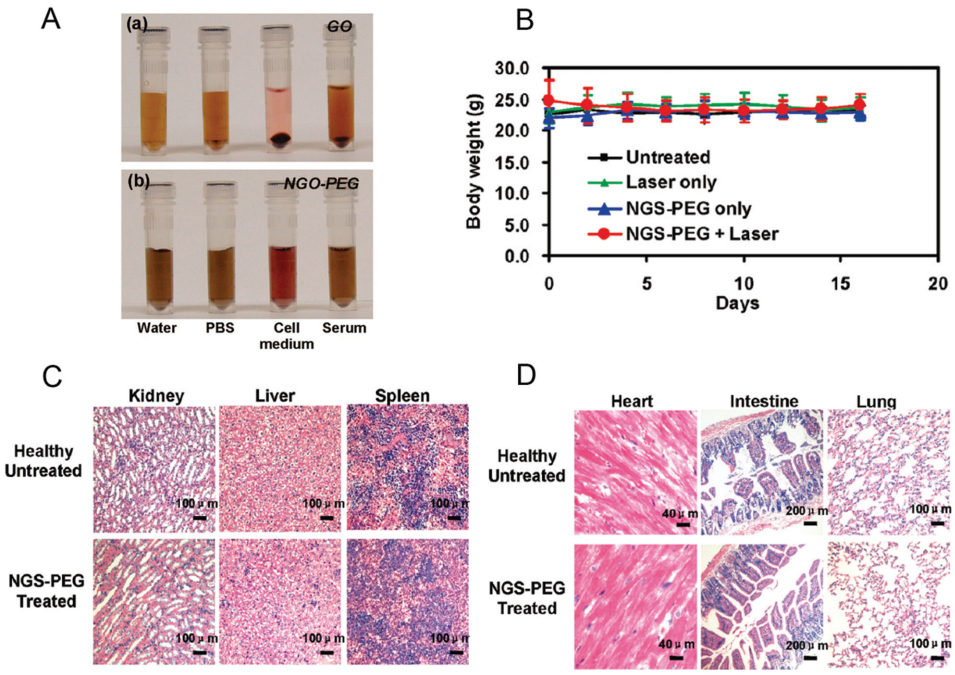

Figure 3. A) Photos of GO and NGO-PEG in different solutions. B) Body weight curves after various treatments indicated. C,D) H&E stained images of major organs. Reproduced with permission: panel (A),[45] Copyright 2008, American Chemical Society; panels (B–D),[47] Copyright 2010, American Chemical Society.

biological properties make them excellent candidates for biomedical applications.

3.2. Utilization of the Electrical Properties of Graphene

As we all know, graphene has higher electrical conductivity due to its unique structure of graphene and strong C–C bonding. Low defect density in the crystal lattice imparts single- layered graphene excellent electrical conductivity.[17] At room temperature, the electrical conductivity of GO is 10^{-1} S/cm. Notably, the electrical conductivity of defect-free monolayer graphene is 10^4 S/cm.[64] It has been demonstrated that impurities trapped between graphene and the substrates and those adsorbed on the graphene surface can significantly affect the electron mobility of suspended graphene.[65] Conductive graphene may significantly improve the electrical conductivity of the composites when being used as fillers with insulating polymer matrix. A variety of factors have been proposed which affect the electrical conductivity and the percolation threshold of the composites, such as the presence of functional groups on graphene sheets, inter-sheet junction, the aggregation of filler, distribution in the matrix, aspect ratio of the graphene sheets, wrinkles and folds, concentration of filers, processing methods, etc.[66] The recent advances made with graphene-based materials mainly utilizing the electrical properties of graphene were highlighted in the following sections.

Metal oxides or hydroxides are mainly used for reinforcing polymers to mimic nacre.[67] While these inorganic nanofillers have high strengths and moduli, they are nonconductive and have high weight densities. Graphene nanosheets are perfect "bricks" for developing the "brick-and-mortar" structures with multifunctionality due to the high strength, thermal stability, and electrical conductivity of graphene. Composite films of CS and rGO sheets with nacre-like layered structure were fabricated by vacuum filtration.[68] The results indicated that the uniform dispersion of rGO nanofillers in the polymer matrices resulted in the high electrical properties of CS/rGO composite films. In comparison to conventional filled polymers, graphene/polymer composites containing extremely lower graphene show superior electrical conductivity. Therefore, much attention has been paid to electrically conductive graphene/polymer composites.

During the solution stirring and sonication, the graphene sheets in solution trend towards forming agglomerates due to van der Waals interactions, which will lead to the formation of big graphene particles and impact on the final composite properties. To prevent the aggregation of graphene during reduction, Hu et al. prepared graphene-coated ultrahigh molecular weight polyethylene (UHMWPE) powders by a two-step process and the resulting composites exhibited a very low percolation threshold and high electrical conductivity (Figure 4C,D).[69] A very flexible nanocomposite film of GO and bacterial cellulose with layered structures was prepared by the vacuum-assisted self-assembly technique.[70] In another example, Sayyar et al. introduced two synthesis procedures to fabricate polycaprolactone/ graphene composites and evaluated the properties of the composites.[71] Ramalingam et al. fabricated the polyvinyl alcohol (PVA)/graphene hybrid nanofibers by electrospinning technique with different concentrations.[72] The results showed an enhanced electrical

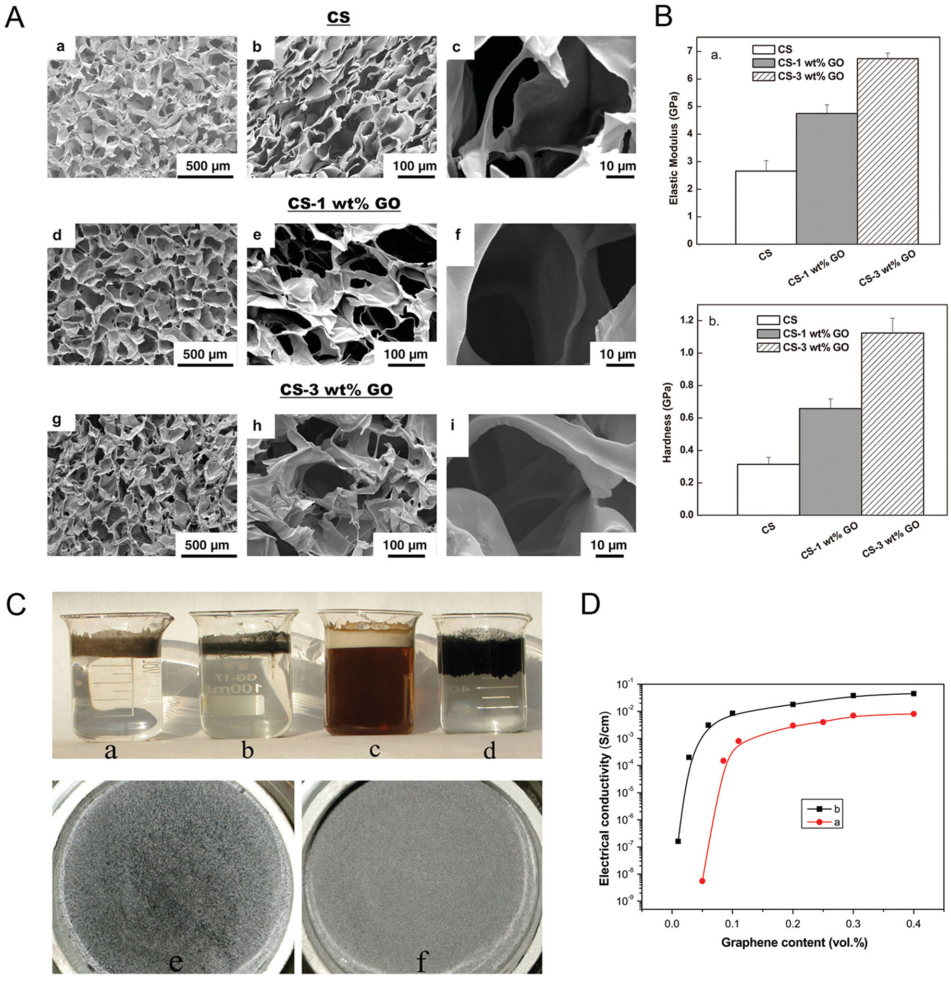

Figure 4. A) SEM images of the different scaffolds. B) Mechanical properties of pure CS and CS-GO scaffolds. C) The color changes in the solutions with different powders before and after reduction. The digital photographs of e) one-step and f) two-step prepared powders. D) Variation of electrical conductivity of the composites derived from a) one-step and b) two-step prepared powders. Reproduced with permission: panels (A,B),[59] Copyright 2011, Elsevier; panels (C,D),[69] Copyright 2012, Elsevier.

conductivity for higher concentration of PVA/graphene hybrid nanofibers compared with pure PVA. Taken together, the graphene-based materials provide a new approach for developing electrically conductive biomaterials.

4. Applications in Regenerative Medicine

4.1. Neural Regeneration

It is still a challenge to recover the full function of injured nerves and repair damaged nerves compared with the treatment of other tissues due to the complexity of the neural system anatomy and function.[73] Neural system will be an ideal breakthrough model in the biomedical applications of graphene. On one hand, the functions of nerve system based on electrical activities and neural cells are electro-active.[74] Clinical diagnostics and treatments often need neuronal stimulation and monitor,[75–77] thus the unique electrical properties of graphene may provide a meaning potential for the therapeutic. On the other hand, graphene can be tailored to match the charge transport requirements of electrical cellular interfacing.[78] Furthermore, chemically stable properties of graphene are beneficial to the integration with neural tissues.

4.1.1. Promoting Stem Cell Differentiation into Neurons

The NSCs are a kind of self-renewing and multipotent cells and determine multilineage differentiation into neurons, astrocytes, and oligodendrocytes. They are the most frequently used stem cell type in neural tissue regeneration and show promising potential for neural regeneration. The recent studies combine various stem cells with graphene-based materials for neural regeneration were illustrated in **Table 1**.

Inducing more human neural stem cells (hNSCs) differentiation towards neurons than glial cells is vital for brain repair and neural regeneration.[79,80] However, many previous studies reported that hNSCs were more like to differentiate to glial cells than neurons without biochemical motifs or co-culturing.[81,82]

Table 1. The most recent strategies frequently combine various stem cells with graphene-based materials for neural regeneration.

Materials	Cell types	Highlights of the study	Ref.
Graphene on a glass substrate	hNSCs	Graphene could induce the differentiation of hNSCs more toward neurons than glial cells.	[83]
3D-GFs	mNSCs	3D-GFs could promote the mNSCs differentiation towards astrocytes and especially neurons.	[39]
Hydrazine-rGO films, Ginseng-rGO films, and GO films	hNSCs	More differentiation of hNSCs into neurons on the hydrazine-rGO and especially the ginseng-rGO films than the GO film.	[84]
Fluorinated graphene	hBMSCs	FG could enhance cell adhesion, proliferation, and neuro-induction of MSCs.	[85]
G and GO	hNSCs	The rGO sheets stimulated by pulsed laser irradiation provided an accelerated differentiation of the hNSCs into neurons.	[88]
CNTs, GO, and graphene	mESCs	GO could significantly promote dopamine neuron differentiation.	[92]

To explore the effect of graphene on NSCs behavior, Park et al. synthesized graphene on a large scale and seeded hNSCs on the substrate, which showed that graphene could induce the differentiation of hNSCs more towards neurons than glial cells.[83] In another study, Li et al.[39] fabricated the 3D porous graphene foams (3D-GFs) and found that the 3D-GFs could promote mouse neural stem cell (mNSC) differentiation towards astrocytes and especially neurons (**Figure 5**A).

Recently, Akhavan et al. explored the differentiation of hNSCs on GO, hydrazine-rGO and ginseng-rGO films, showing more hNSCs differentiated into neurons on the hydrazine-rGO and especially the ginseng-rGO films than on the GO films.[84] The accelerated differentiation on the rGO films was attributed to their higher capability for electron transfer. Meanwhile, the better differentiation on the ginseng-rGO films was assigned to more hydrophilicity, higher biocompatibility, and the π–π attachment of ginsenoside molecules on the surface of the reduced sheets. Furthermore, an effective and self-organized differentiation of hNSCs into neurons was realized by the pulsed laser stimulation of the cells on graphene films.[85]

It is interesting to consider functionalized graphene such as fluorinated graphene (FG) as the strong polarity of the carbon-fluorine bond, which is expected to induce biological responses.[86,87] For instance, Wang et al. used FG sheets as the scaffold for human bone marrow derived mesenchymal stem cell (hBMSC) growth.[88] Morphological changes indicated that FG could enhance the neural differentiation of hBMSCs and the effect could be further enhanced with neuron inducer. Moreover, the stem cells could be controllably patterned on fluorinated graphene and could be induced to neuronal lineage in the absence of chemical inducer (Figure 5B).

Many studies have demonstrated that transplanting dopamine neurons is a promising therapy.[89] Embryonic stem cells (ESCs) differentiation into dopamine neurons in vitro offers a useful tool for the genetic profile underlying dopamine neuron development and derivation for transplantation.[90] However, it is still a challenge to efficiently differentiate ESCs into dopamine neurons.[91] Given the existing evidence of nanomaterials on stem cell proliferation and differentiation, Yang et al. investigated the effect of CNTs, GO, and graphene on the differentiation of mouse embryonic stem cells (mESCs).[92] They found that only GO could significantly accelerate dopamine neuron differentiation after induction and further accelerate dopamine neuron-related gene expression compared with the other groups.

4.1.2. Sustaining Neuronal Survival and Promoting Neuronal Outgrowth

Emerging concerns on graphene are its biocompatibility and how targeted cells respond to it. Relatively few studies intended to find the interactions of graphene or its derivatives with the cells, whereas very few reports on neural system. Li et al. tried

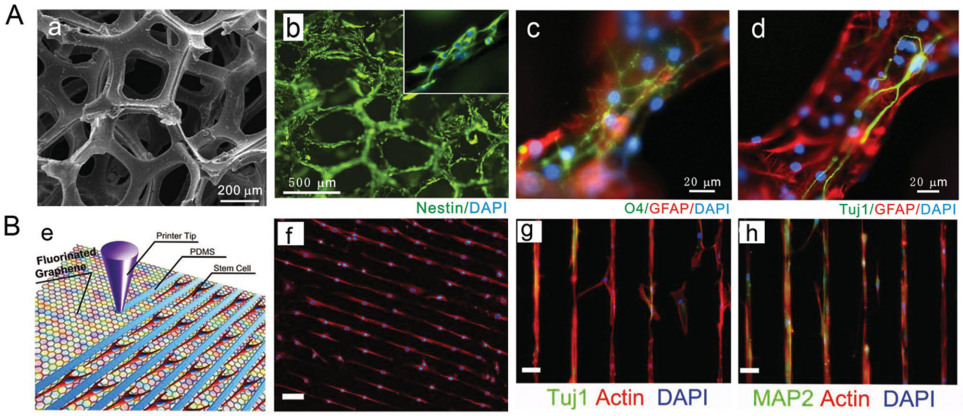

Figure 5. A) SEM of 3D-GFs at a) low magnification. b) Fluorescence images of NSCs proliferated on 3D-GFs. c,d) Representative fluorescence images of differentiated NSCs under differentiation conditions. B,e) Schematic drawing of patterning MSCs. f) The aligned growth of stem cell on FG with printed PDMS pattern. g,h) MSCs preferentially attached on the FG strips and their F-actin aligned (red) and expressed neural specific markers-Tuj1 and MAP2 (green). Reproduced with permission: panel (A),[39] Copyright 2013, Nature Publishing Group; panel (B),[88] Copyright 2012, John Wiley and Sons.

to explore how neurites were affected by graphene in a mouse hippocampal culture model. The results showed that graphene could greatly promote neurite sprouting and outgrowth to the maximal extent during the early developmental phase.[74] Similarly, Bendali et al. investigated the survival of adult retinal neurons directly contacting with bare graphene and found that adult neurons could successfully survive and grow neurites.[93]

Many physiological functions include charge or electrical transfer, particularly at the cell membrane interfaces. Using an applied current, i.e., to stimulate neuron can mediate this process.[39,94] Electroactive scaffolds that can transmit applied electrical stimuli are very important for neural tissue regeneration. Graphene-heparin/poly-L-lysine polyelectrolytes were assembled via the self-assembly of aqueous colloidal graphene onto 2D surfaces and 3D electrospun nanofibers.[95] The employed layer by layer (LBL) coating technique enabled the electro- and biofunctionalization of nano- to microscale scaffolds with complicated internal structures. The in vitro results indicated that both 2D and 3D graphene-polyelectrolyte multilayers supported neuron cell attachment and neurite outgrowth.

4.1.3. Improving Neural Electrical Performance after Electrical Stimulation

Developing conductive platform that introduces external electrical stimuli to NSCs is a new trend because electrical stimulation can affect the migration, proliferation, and differentiation of NSCs.[96,97] Electrically conductive scaffolds can be fabricated by combination of conductive polymers and carbon-based materials such as CNTs, graphite, and graphene.[98] Among these conductive materials, graphene is an emerging conductive material because of its extraordinary properties such as electrical conductivity, exceptionally high specific surface area, and electrochemical potential window.[99] It has been demonstrated that graphene-based substrates are not only biocompatible but also can improve neural cell growth. When investigate the effects of graphene on the electrical activity of neuronal networks, the study of graphene for tissue regeneration has provided further outstanding surprises.

Park and colleagues investigated the electrical neural activity of the differentiated cells from NSCs utilizing graphene films as a stimulating electrode.[83] They found the cells after electrical stimuli exhibited over 60–70% fluorescence intensity increase, which indicated an increased calcium level inside the cell. This result presented that graphene films could be used as a neural-stimulation electrode and the neural activity of the differentiated cells was proved by electrical stimulation using the graphene electrode. In addition, Li et al. conducted research on the 3D-GFs scaffold as a conductive platform for cell electrical stimulation, which showed the 3D architecture of GFs could provide an enormous interface and 3D multiplexing and could effectively improve the electrical stimulation performance of conductive scaffold.[39]

When graphene is used for stem cell based therapy, the cells after stem cell differentiation should preserve normal or even enhanced activities and form functional connections from each other. Tang et al. evaluated the effects of graphene on the functional formation and neural activities in the assembly of neural networks in NSCs culture.[100] Graphene was developed as an electrode to observe the cell response to the electrical stimulation. With electrical stimuli, the cells exhibited approximately 30% fluorescence intensity increase, which clearly implied that the electrical stimulation could be transferred to the neurons by conductive graphene.

4.1.4. Patterned Graphene Substrates for Neuron Growth

In order to find out the in vivo neural circuitry both for fundamental neurophysiology and prosthetic applications, many studies have been attempted to culture neurons according to an ordered pattern.[101] Patterned neurons covering modified conductive materials are being sought to build multi electrode arrays by controlling the neural activity at defined points.[102] Notably, patterned graphene is shown to be a suitable substitute for the current biocompatible conductive materials.

Reduced graphene oxide nanoribbon (rGONR) grid was deposited on the surface of a SiO_2 film including TiO_2 nanoparticles to use as a photocatalytic stimulator for the differentiation of hNSCs into patterned neural networks.[103] The rGONR grid exhibited patterned proliferations of hNSCs and higher neural differentiation as compared with the random differentiations on quartz and rGO substrates. Nanotopographical features prepared using arrays of silica microbeads had been shown to lead to the enhancement of axonal growth of hippocampal neurons.[104] Thus, Solanki and colleagues prepared graphene-silica nanoparticle hybrids by coating GO nanosheets on the surface of 300 nm silica nanoparticles (SiNPs). The nanotopographical features modified with GO provided instructive physical cues and led to promoted neuronal differentiation of hNSCs along with significant axonal alignment (**Figure 6**).[105] In another study, Lorenzoni et al. presented a straightforward fabrication technique to get patterned substrates for enhancing ordered neuron growth.[106] They patterned single layer graphene on technologically interesting substrates by using large area fabrication technique, which resulted in notably higher alignment for neuron adhesion and growth.

4.1.5. Graphene for Neural Interfaces

Recent studies have generated extensive interest in the creation of interfaces between external devices and neurons to supplement or restore the function of the neural system lost during disease or injury. The neural interfaces should relay the electrochemical signals between a soft, wet tissue and a stiff, dry electrode because biological cells are excited by ionic potentials. Graphene is an attractive candidate for bioelectronic applications for its remarkable physical and chemical properties. The field-effect transistor (FET) performance of graphene surpasses most semiconductors due to the high charge carrier mobility in graphene. Graphene has good biocompatibility and chemical stability, which is beneficial to integrate with biological systems. Moreover, the facile integration of graphene electronics with flexible substrates is important for the development of biomedical implants with reduced tissue damage and scarring.[107]

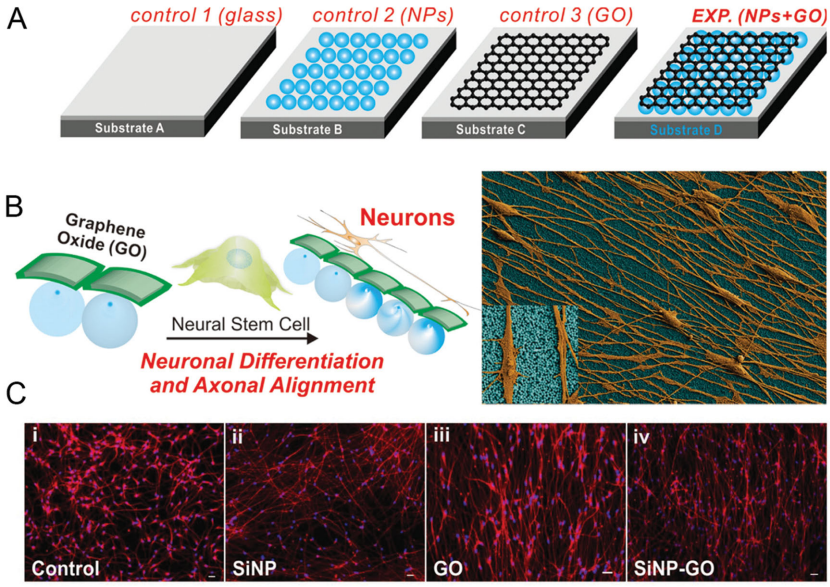

Figure 6. A) Different control and experimental conditions for differentiating hNSCs into neurons. B) hNSCs cultured and differentiated on Substrate D having a monolayer of NPs coated with GO. C) Differentiated hNSCs are immunostained with TuJ1 (red). Adapted with permission.[105] Copyright 2013, John Wiley and Sons.

Interfacing of living cells and tissue with solid-state electronic devices has mostly depend on conventional silicon technology.[108] However, this technology has some drawbacks, such as a relatively high electrical noise and its limited stability in aqueous environments.[109] Thus, solution-gated field-effect transistors (SGFETs) based on graphene are developed as sensing devices.[107] For example, Cohen-Karni et al. fabricated graphene FETs and combined graphene and nanowire FETs interfaced with embryonic-chicken cardiomyocytes by using a single transistor on exfoliated graphene.[110] Graphene FETs conductance signals collected from spontaneously beating cardiomyocytes generated well-defined extracellular signals with signal-to-noise ratio routinely >4, which exceeded representative values for other planar devices. Along similar lines, Hess et al. reported on arrays of graphene-based solution-gated field-effect transistors (G-SGFETs) for the detection of the electrical activity of electrogenic cells. They successfully resolved and tracked the action potentials of cardiomyocyte-like HL-1 cells across the transistor array.[107] Thus, the low noise of G-SGFETs and the large transconductive sensitivity of these devices could make graphene SGFETs better than most of the known devices in terms of signal-to-noise ratio.

A graphene/polyethylene terephthalate film stimulator and a non-contact electric field stimulation protocol were developed to promote the cell-to-cell interactions,[111] which showed that weak electric field stimulation could promote new cell-to-cell coupling and strengthen existing cell-to-cell coupling due to an altered regulation of the endogenous cytoskeletal proteins. To make the implantable bioelectronic devices possess excellent electrochemical characteristic, Tian et al. doped GO into poly(3,4-ethylene dioxythiophene) (PEDOT) to form a composite film by electrochemical deposition for electrode site modification.[112] As a consequence, not only the enlargement of efficient surface area, but also the development of impedance, charge storage capacity, and charge injection limit contributed to the excellent electrochemical performance of the PEDOT/GO films. Therefore, as electrode-tissues interface, the graphene-based materials open a new gate for tissue engineering and implantable electrophysiological devices.

4.2. Bone Regeneration

Bone is a dynamic, rigid, highly vascularized tissue with a unique capacity to remodel and heal without leaving a scar.[113] However, remodeling large bone defects caused by severe trauma, congenital malformations, tumors, and nonunion fractures is limited.[114] Bone tissue engineering offers a promising new approach for clinical use, in which autologous cells are co-cultured with biomaterial scaffolds.[115]

Before talking about the role and probable interactions of graphene in bone tissue regeneration, we need to recall the typical types of cells exist in human bone-related biological environment. One type of the important recruited cells is mesenchymal stem cell (MSC) which plays a crucial role in the natural process of new bone formation by differentiating into osteoblasts.[116] How to direct the differentiation of stem cells towards osteoblast lineage still remains a challenge in bone regeneration. Another cell type is osteoblast which first adheres on the extracellular matrix (ECM) surface to initiate bone formation, and then they spread and proliferate to cover the ECM surface.[117] Other cells include fibroblasts and sarcoma cells can also participate in bone regeneration.[118]

4.2.1. Supporting In Vitro Osteogenic Differentiation of Mesenchymal Stem Cells

Current tissue regeneration approaches combine various biomaterials with cells to offer biological substitutes that can repair and eventually restore tissue functions.[119,120] Various biomaterials have been developed for transplantation of stem cells and their specific differentiation into bones, cartilages, and muscles.[119,121–123] One of the vital aims for bone regeneration is to direct the proliferation of stem cells and to promote their differentiation in a controlled manner with the use of osteogenic inducers and growth factors.[124,125] To repair and regenerate bone tissue, the use of graphene-based materials and stem cells is being studied and the effective promotion of the osteogenic differentiation of MSCs is one of the core issues in this field. Some of the examples were summarized in **Table 2**.

In a primary study by Kalbacova et al., human mesenchymal stem cells (hMSCs) cultured on the large single layer graphene showed spindle-shape morphology and were homogenously dispersed on the surface, which indicated that graphene was

Table 2. The most recent strategies frequently combine various stem cells with graphene-based materials for bone regeneration.

Materials	Cell types	Highlights of the study	Ref.
Single layer graphene produced by CVD	hMSCs	Graphene is promising for bone reconstructive surgery with an increased likelihood of inducing MSC differentiation into the osteoblast lineage.	[126]
Graphene coated with PMMA	hMSCs	Graphene could accelerate the differentiation of hMSCs at a rate comparable to differentiation under the influence of BMP-2.	[127]
Graphene and GO films	hMSCs	Graphene and GO are demonstrated to be effective preconcentration platforms for accelerated stem cell growth and differentiation.	[128]
GO-coated Ti substrate	hMSCs	The BMP-2-adsorbed Ti/GO substrates could significantly enhance in vitro osteogenic differentiation of hMSCs and induce more robust bone formation	[129]
Graphene nanogrids	hMSCs	rGONR grids showed the fastest osteogenic differentiation of the hMSCs reported up to now in short time of 7 days in the presence of chemical inducers.	[130]
Self-supporting graphene hydrogel films	rBMSCs	The SGH film alone is able to stimulate osteogenic differentiation of stem cells without the need for any additional inducer.	[131]
Graphene-coated plates	gMSCs	GO supports proliferation and osteogenic differentiation of gMSCs without the addition of any glucocorticoid or specific growth factors.	[132]
3D graphene foams	hMSCs	3D graphene foams can support the attachment and viability of hMSCs, and induce spontaneous osteogenic differentiation.	[133]
Graphene-incorporated CS substrata	hMSCs	Nanotopographic cues of the substrata promoted adhesion and differentiation of hMSCs.	[134]
GO/PLL composite films	rBMSCs	GO/PLL composite film could not only support the growth of MSCs with a high proliferation rate, but also could accelerate the osteogenic differentiation of MSCs.	[135]
PLLA nanofibrous scaffolds containing CNT and graphene	mBMSCs	Graphene showed stronger effect on promoting osteogenic differentiation of BMSCs and inducing osteogenesis in vivo than CNT.	[136]
GO-CaP nanocomposites	hMSCs	GO-CaP nanocomposites significantly facilitated the osteogenesis of hMSCs and further enhanced calcium deposition.	[137]

promising for inducing hMSCs differentiation into the osteoblast lineage.[126] Nayaket et al. went a step further and indicated that graphene could accelerate osteogenic differentiation of hMSCs into bone cells at a rate comparable to differentiation under the influence of bone morphogenetic protein-2 (BMP-2) (**Figure 7**).[127] Similarly, Lee et al. reported that MSCs cultured on graphene were more osteogenic and deposited more minerals compared with GO and polydimethylsiloxane (PDMS) substrates.[128] Furthermore, they were the first to elucidate that the origin of the osteogenic differentiation could be contributed to the ability of graphene to act like a preconcentration platform for *β*-glycerolphosphate and dexamethasone. In another study, La et al.[129] found that the GO-coated Ti substrate (Ti/GO) enabled enhanced adsorption and sustained release of BMP-2 while maintaining the intrinsic bioactivity of the protein. The BMP-2-adsorbed Ti/GO substrates could significantly enhance in vitro osteogenic differentiation of hMSCs and induce more robust bone formation than the BMP-2-adsorbed Ti substrates, which was likely attributed to the higher conformational stability, higher bioactivity, and the increased local concentration of BMP-2 on the Ti/GO- surface.

The nanostructures of graphene such as graphene nanoribbons were fabricated and used as selective 2D templates in accelerated proliferation and differentiation of hMSCs.[130] The rGONR grid exhibited the fastest osteogenic differentiation of hMSCs with chemical inducers in a short time of 7 days. In addition, the amount of differentiation after 7 days was found to be ≈2.2-fold greater than the differentiation on rGO sheets. The greatly accelerated differentiation on the rGONR grids was attributed to both physical stresses induced by the surface topographic features of the nanogrids and the capability of the rGONR grids in high adsorption of the chemical inducers.

On the other hand, a simple form of graphene-based bulk material-self-supporting graphene hydrogel (SGH) film was used as an appropriate platform to investigate the intrinsic properties of graphene both in vitro and in vivo.[131] The results indicated that the SGH film alone could stimulate osteogenic differentiation of rat bone marrow stromal stem cell (rBMSCs) without the need for any additional inducer (**Figure 8**). In a similar study, the graphene's effect on the growth and differentiation of goat adult mesenchymal stem cells (gMSCs) was also investigated.[132] In recent years, the 3D graphene foams were developed and employed in bone regeneration field. Crowder et al. fabricated 3D porous graphene foams by growing graphene on a 3D nickel scaffold and employed the 3D graphene foams as culture substrates for hMSCs.[133] These results provided evidences that graphene materials could promote the adhesion and viability of MSCs and induce spontaneous osteogenic differentiation.

The graphene-based composite materials have also been applied for bone tissue regeneration in combination with MSCs. For example, Kim et al. fabricated graphene-incorporated CS substrata and found that graphene with unique characteristics of the nanoscale topographical cue and its secondary effects such as stiffness and roughness promoted adhesion and differentiation of hMSCs.[134] A new type of GO/poly L-lysine (PLL) composite films was synthesized via LBL assembly for

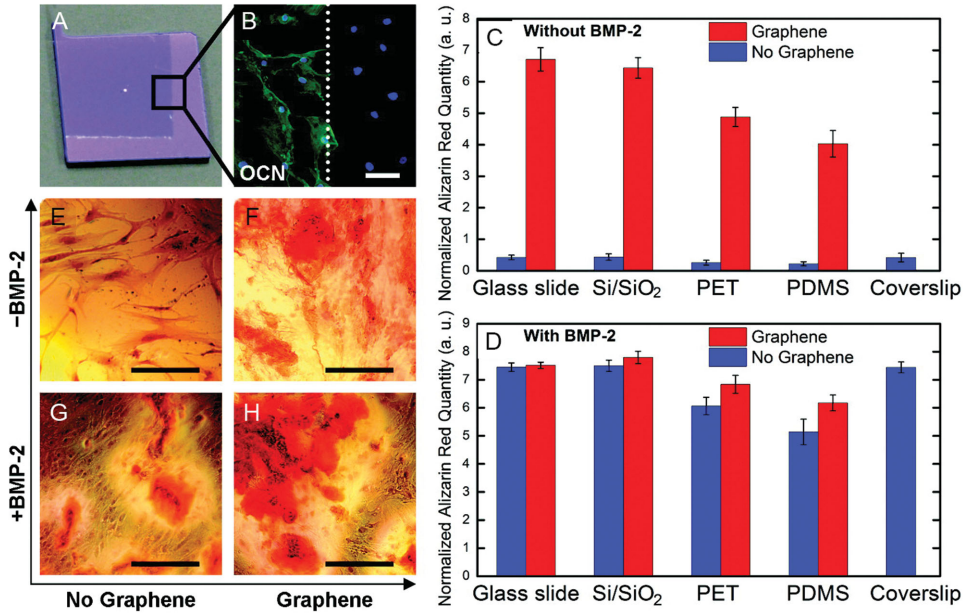

Figure 7. Graphene accelerates osteogenic differentiation. A) Optical image of partially graphene-coated Si/SiO_2 chip. B) Osteocalcin (OCN) marker showing bone cell formation on the same chip only positive control. E–H) polyethylene terephthalate (PET) substrate stained with alizarin red. e) PET without BMP-2 and without graphene; f) PET without BMP-2 and with graphene; g) PET with BMP-2 and without graphene; h) PET with both BMP-2 and graphene. Adapted with permission.[127] Copyright 2011, American Chemical Society.

adhesion, proliferation, and differentiation of the MSCs.[135] Notably, the GO/PLL composite film was able to promote the growth of MSCs with a high proliferation rate, and also enhance the osteogenic differentiation of MSCs. The results suggested that the high pre-concentration capacity of the GO/PLL films for osteogenic inducers could play an important part in the significant enhancement of osteogenic differentiation of MSCs. Similarly, poly(L-lactide) (PLLA) nanofibrous scaffolds containing carbon nanomaterials, such as CNT and graphene were fabricated by Duan et al.[136] In a recent study, Tatavarty et al. synthesized nanocomposites consisting of oblong ultrathin plate shaped calcium phosphate (CaP) nanoparticles and GO microflakes.[137] The GO-CaP nanocomposites significantly facilitated the osteogenesis of hMSCs and further enhanced calcium deposition.

Knowledge on the effects of graphene is limited and studies on the contribution of graphene to bone tissue regeneration are still quite new until now. Within the limitation of the present investigation, we believe that the applications of graphene-based materials for bone tissue regeneration may have a promising future, and further research will realize its potential.

4.2.2. Modulating the Directional Growth and Activity of Osteoblasts

The effect of graphene-based materials on cellular behavior is vital for enabling a range of bone applications. However, due to the complexity of graphene surface states and cell responses, it is a great challenge to control cellular behaviors on graphene and its derivatives.

Mahmood et al. correlated the positive effects of graphitic nanomaterials with different structures (MWCNTs and graphene sheets) on the process of bone cell mineralization, which indicated that graphene did not have any toxic effects on osteoblasts while graphene coated substrates showed more osteoblasts adhesion than the substrate alone.[138] Kanayama and colleagues modified a bio-safe collagen scaffold with GO and rGO and assessed the biological effects of GO and chemically synthesized rGO films.[139] rGO strongly enhanced calcium absorption and alkaline phosphatase (ALP) activity, indicating that rGO was effective for osteogenic differentiation. In another study, graphene nanoplatelets were added as reinforcement to UHMWPE by electrostatic spraying,[140] which showed that the cytotoxicity of graphene nanoplatelets to osteoblast was dose-dependent and was also affected by the agglomeration of particles.

In the last few years, composite materials containing graphene derivatives and inorganic components such as hydroxyapatite (HA), are considered as potential candidate scaffolds for bone regeneration due to their bone compatibility and remarkable mechanical properties. For example, Kim et al. fabricated self-standing GO/graphene-$CaCO_3$ composites composing of vaterite microspheres, the most unstable crystalline polymorph of $CaCO_3$ was wrapped and interconnected by GO (or graphene) networks, showing high viability of osteoblast cells with elongated morphology.[141] rGO reinforced hydroxyapatite nano-tube composites were fabricated by Baradaran et al., and the in vitro results showed that the addition of the rGO could promote osteoblast adhesion and proliferation.[142] In a related application, a convenient one-pot hydrothermal strategy was developed to synthesize graphene nanosheets/HA nanorod composites.[143]

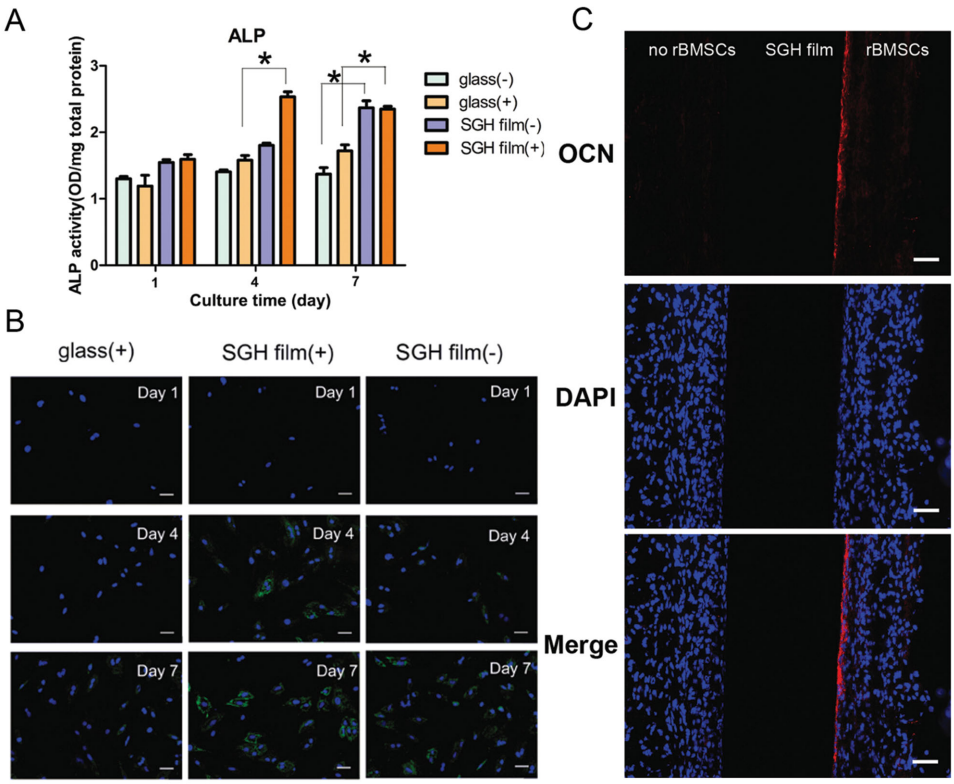

Figure 8. A) ALP activity of cells growing on glass and on the SGH film. (–): growth medium. (+): osteogenic medium. B) Immunofluorescence staining for OCN of cells growing on glass and on the SGH film. C) Immunofluorescence staining for OCN of the SGH film at 4 weeks after implantation. Adapted with permission.[131] Copyright 2013, John Wiley and Sons.

For the first time, Li et al. developed GO and CS functionalized GO as templates to fabricate HA using a facile solution-based in situ synthesis method and successfully prepared GO-HA and CS-GO-HA nanocomposites.[144] Both of the nanocomposites displayed a high cell proliferation rate and the CS-GO-HA composite showed greatly higher cell viability and ALP activity compared with GO-HA. Similarly, a genipin-cross-linked CS/GO composite film was fabricated via a solution casting method and the in vitro results showed that the composite films were suitable for the proliferation and adhesion of MC3T3-E1 cells.[145]

4.3. Cartilage Regeneration

The articular cartilage is an avascular, non-nervous, and elastic tissue consisting of sparsely distributed chondroblasts embedded within dense ECM, which is composed of collagen fibers and abundant ground substance rich in proteoglycan and elastin fibers.[146] The chondrogenic differentiation of stem cells is conventionally achieved by culturing cells in pellets.[147] However, pellets provide low cell-ECM interaction and diffusional limitation of protein transforming growth factor-β3 (TGF-β3) may occur inside the pellet, which may limit the chondrogenic differentiation of stem cells. To overcome such problems, GO sheets were used to adsorb fibronectin (FN) and TGF-β3 and were incorporated in pellets for chondrogenic differentiation of human adipose-derived stem cells (hASCs) in pellets.[148] The hybrid pellets of hASC-GO accelerated the chondrogenic differentiation of hASCs by adding the cell-FN interaction and supplying TGF-β3 effectively (**Figure 9**). The use of GO to accelerate chondrogenic differentiation of stem cells may open a new direction in cartilage regeneration.

4.4. Skeletal Muscle Regeneration

Skeletal muscles are contractile tissues composed of bundles of highly oriented and dense muscle fibers. Traumatic injury or tumor ablation or functional damage due to myopathies may lead to severe functional damage. Various muscle transplantations have been developed to partially repair the loss of muscle mass and their function. However, the engineering of skeletal muscle tissue is still a scientific challenge.[149,150] The myotube formation on GO and rGO was evaluated in vitro. Myogenic differentiation was remarkably enhanced on GO, which was attributed to more oxygenous functional groups and surface roughness that influenced the adsorption of serum proteins.[151] The results suggested that GO could accelerate myogenic differentiation, indicating potential applications in skeletal muscle regeneration.

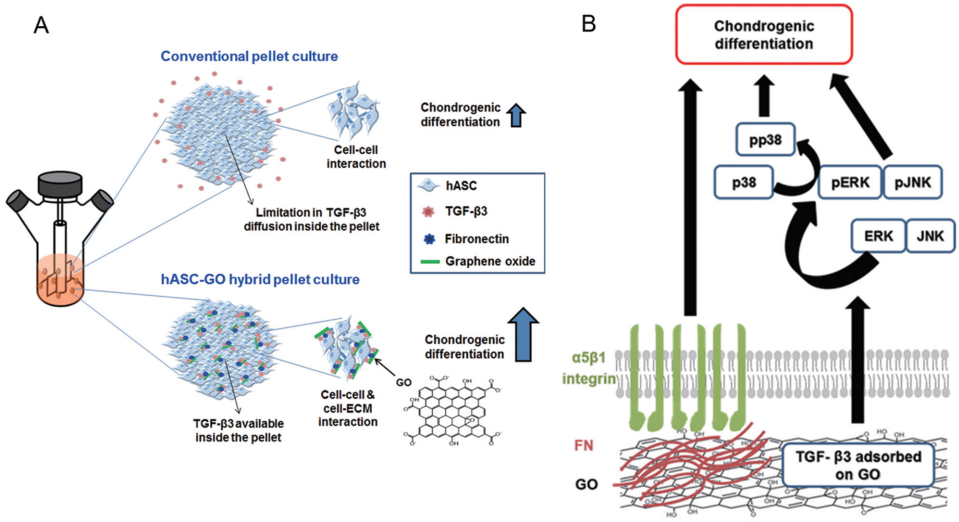

Figure 9. A) A schematic diagram describing the enhancement in chondrogenic differentiation of hASCs using GO. B) A schematic diagram of the underlying mechanisms describing the cell signaling in the enhanced chondrogenic differentiation in the hASC-GO hybrid pellets induced by TGF-β3 and FN. Adapted with permission.[148] Copyright 2014, John Wiley and Sons.

4.5. Cardiac Regeneration

It has been demonstrated that MSCs have great potential to repair heart diseases. However, published clinical trial experiences of MSCs as cardiac therapy are limited as MSCs hardly differentiate into cardiomyocytes in vivo.[152] The transplantation of cardiomyogenically differentiated MSCs has been shown to greatly improve myocardial contractility.[153] Thus, many researchers try to utilize 5-azacytidine to direct MSCs towards the cardiomyogenic lineage.[154] However, 5-azacytidine-treated MSCs are not clinically appropriate because it interferes with normal cell activity by suppressing deoxyribonucleic acid methylation,[155] Thus, other methods for MSC differentiation into the cardiomyogenic lineage need to be developed for clinical stem cell therapies for myocardial infarction.

Park et al. firstly proposed that graphene could upregulate the expressions of cardiomyogenic genes of MSCs and found that graphene promoted MSCs differentiate into the cardiomyogenic lineage without any exogenous chemical inducers.[156] The enhanced cardiomyogenic differentiation of MSCs cultured on graphene might be due to the upregulation of cell signaling molecules and gene expressions of ECM proteins. This finding indicated that graphene-based materials might be a novel platform for the cardiomyogenic differentiation of MSCs.

4.6. Skin Regeneration

Skin is the biggest organ of the body and it protects the human body from surrounding environment. Over the years, many approaches have been developed to make skin replacements that mimic human skin, and many natural and synthetic materials have been designed to generate artificial skin tissues.[157] To combine the advantageous features of graphene and CS for use in wound healing, Lu et al. doped a few layers of graphene on the electrospun CS-PVA nanofibers.[158] The in vivo studies showed that the graphene-containing scaffolds promoted wound healing at a faster rate in comparison to other groups in the rabbit and mice models. It was suggested that the free electron of graphene did not influence the eukaryotic cell multiplication but suppressed the multiplication of prokaryotic cells, thus impeding the proliferation of microbes and benefiting wound healing. This study firstly made it possible to directly use graphene in wound healing, and might promote the development of biomedical applications of graphene. In a related application, collagen-fibrin (CF) biocomposite films incorporated with GO (CFGO) were prepared for would healing.[159] The existence of GO enhanced the mechanical strength of collagen/fibrin films. Faster wound healing was found in CFGO treated rats compared with those without GO, which indicated that graphene-based materials enhanced wound healing and might be used on more clinical wounds of various animal models before their applications on human beings.

4.7. Adipose Tissue Regeneration

Adipose tissue is the key component necessary for soft tissue reconstruction. Restoring adipose tissue is a strong clinical need because contour defects not only affect patients cosmetically but also impair functions.[160,161] The effects of graphene and GO substrates on the adipogenic differentiation of MSCs were investigated by Lee et al.,[162] which showed that the differentiation to adipocytes was significantly suppressed on graphene because insulin was denatured upon π–π adsorption on graphene. Conversely, GO greatly enhanced adipogenic differentiation due to its high affinity for insulin. These findings suggested that GO were effective preconcentration platforms

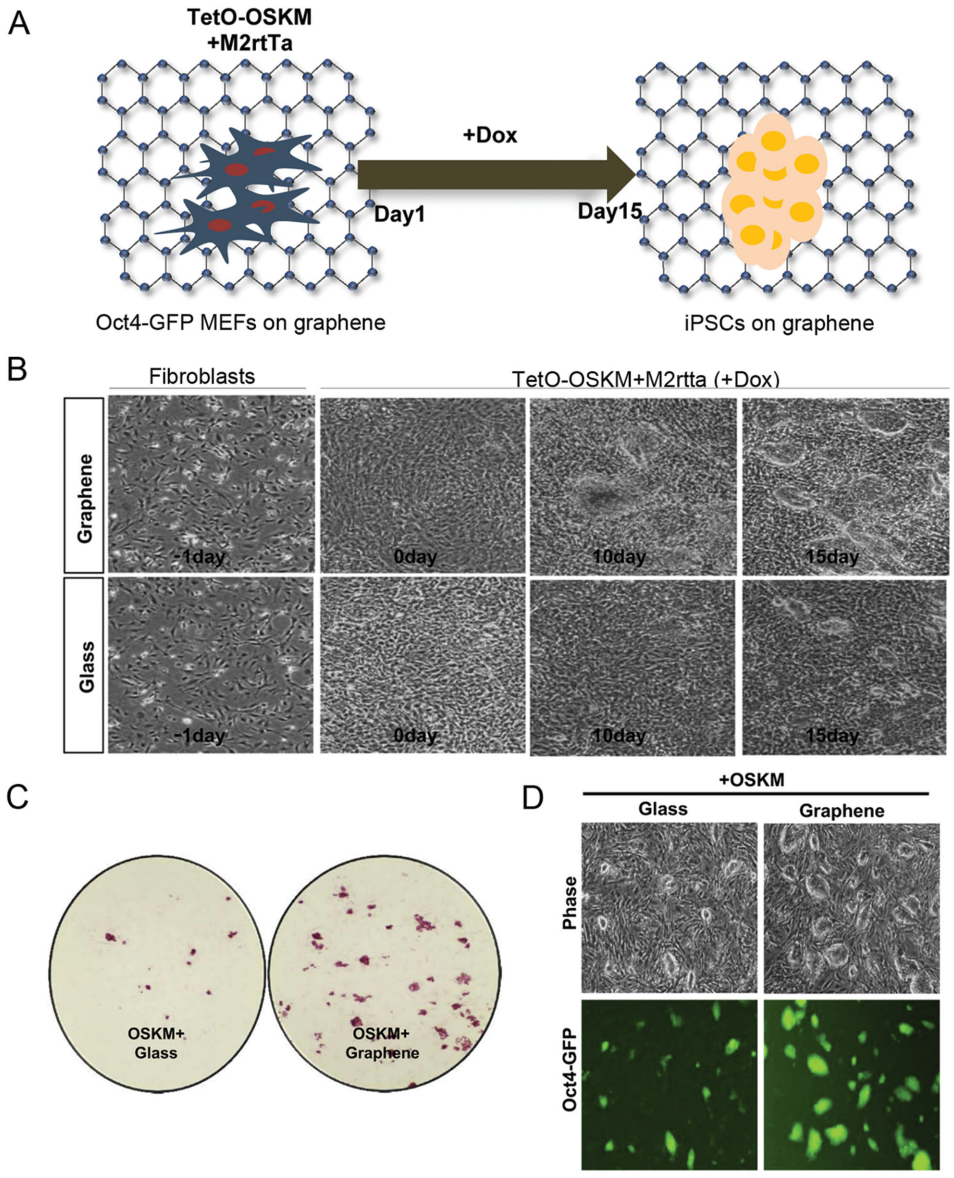

Figure 10. A) Schematic drawing of cell reprogramming on the graphene-based substrate. B) Representative images of the generated iPSCs on glass and graphene-based surface. C) Representative images of alkaline phosphatase staining of the generated iPSCs. D) GFP expression of iPSCs colonies generated from Oct4-GFP KI fibroblasts. Adapted with permission.[172] Copyright 2014, Elsevier.

for accelerated stem cell differentiation into adipocytes through molecular interactions.

4.8. Effects of Graphene on Induced Pluripotent Stem Cells

In recent years, the ability to reprogram an adult somatic cell into a pluripotent stem cell is a major breakthrough in regenerative medicine.[163–165] Patient-specific cell therapies can be realized with induced pluripotent stem cells (iPSCs) derived from somatic cells, which avoids ethical concerns and immune rejection issue. In addition, iPSCs have unlimited expansion potential, which makes them an excellent cell source for tissue regeneration. However, before using iPSCs as a cell source, some critical issues need to be cleared, such as the various differentiation lines of iPSCs and suitable differentiation stage of the cells.[166] iPSCs were cultured on the surface of graphene- and GO-coated substrates to evaluate their proliferation and differentiation.[167] Compared with iPSCs cultured on the glass surface and graphene surface, iPSCs on the GO surface attached and proliferated at a faster rate. The differentiation into ectoderm and mesoderm was similar for iPSCs cultured on both graphene and GO. However, graphene hampered the iPSCs differentiation towards the endodermal lineage whereas GO promoted the endodermal differentiation.

Cell reprogramming is an approach to generate ESC-like cells from somatic cells by ectopic expression of defined factors.[168] However, it is very hard to generate iPSCs and cell reprogramming is a random procedure in which some problems must be solved to make the cells pluripotent.[169] It has been demonstrated that inhibiting epithelialization can suppress the epigenetic reprogramming.[170] In addition, previous studies showed that microtopography substrate could affect the mesenchymal-to-epithelial-transition (MET) and thereby could improve reprogramming efficiency.[171] In a recent study, Yoo et al. reported that graphene could promote the reprogramming of mouse somatic fibroblasts into induced iPSCs.[172] They showed that graphene could dramaticlly enhance the cellular reprogramming efficiency by inducing MET that affected H3K4me3 levels, which indicated that graphene substrate directly regulated dynamic epigenetic changes associated with reprogramming (**Figure 10**). The results revealed that graphene could promote cell fate changes associated with reprogramming and provide an efficient tool for various applications in iPSCs-based regenerative medicine.

5. Conclusion and Future Prospects

The applications of graphene in regenerative medicine have grown rapidly in the past few years. Some significant and exciting progresses have been made in this field. These preliminary preclinical studies are encouraging; however, some remaining challenges are still required to be addressed to realize future clinical applications.

Graphene-based materials have shown excellent electrical conductivity and improved mechanical properties. However, preventing the aggregation of graphene in solution and the homogeneous distribution of graphene nanosheets in the matrix remain to be addressed. In order to make further progress, novel approaches have to be developed to distribute graphene nanosheets homogeneously in the matrix and prevent the graphene aggregation. In addition, the use of biocompatible polymers in the shape of 3D scaffolds containing graphene with remarkable electrical and mechanical properties may provide an update on the development of designing 3D organs in the future.

Some promising findings using graphene-based materials for stem cell research are presented in this article. Nevertheless, little is known about the cellular mechanisms and signaling pathways involved in the progress of stem cell differentiation till now. Research in this field is still in its early stage and much more studies are required to uncover the mechanism in order to guide stem cell differentiation to different designated lineages. Graphene, as a conductive material, can play important roles in neural interface engineering. While many researchers have tried some in vitro methods for electrical stimulation, clinical applications of electrical stimulation using graphene-based materials have not succeeded. More studies are needed to completely understand and simultaneously compare the effects of various conditions of electrical stimulation on neurons. In addition, the mechanisms caused by electrically stimulated cells need further investigation, and in vivo electrical stimulation using implanted conductive graphene-based materials must be carried out for deeper understanding and efficacy evaluations in nerve regeneration.

Although concerns about cytotoxicity may be mitigated by chemical functionalization of graphene, the potential long-term toxicity is still the major challenge in this area. Thus, more toxicity studies using in vivo animal models are needed to investigate the safety and biocompatibility of graphene-based materials. In summary, in spite of various unresolved issues and challenges, the use of graphene-based materials may pave the way for a true breakthrough in future studies of regenerative medicine.

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